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Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite-sensitive transcription repressor

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Summary

Production of nitric oxide (NO) and nitrous oxide (N_2O) by ammonia (NH_3)-oxidizing bacteria in natural and man-made habitats is thought to contribute to the undesirable emission of NO and N_2O into the earth's atmosphere. The NH_3-oxidizing bacterium *Nitrosomonas europaea* expresses nitrite reductase (NirK), an enzyme that has so far been studied predominantly in heterotrophic denitrifying bacteria where it is involved in the production of these nitrogenous gases. The finding of *nirK* homologues in other NH_3-oxidizing bacteria suggests that NirK is widespread among this group; however, its role in these nitrifying bacteria remains unresolved. We identified a gene, *nsrR*, which encodes a novel nitrite (NO_2^-)-sensitive transcription repressor that plays a pivotal role in the regulation of NirK expression in *N. europaea*. NsrR is a member of the Rrf2 family of putative transcription regulators. NirK was expressed aerobically in response to increasing concentrations of NO_2^- and decreasing pH. Disruption of *nsrR* resulted in the constitutive expression of NirK. NsrR repressed transcription from the *nirK* gene cluster promoter (P_{nir}), the activity of which correlated with NirK expression. Reconstruction of the NsrR-P_{nir} system in *Escherichia coli* revealed that repression by NsrR was reversed by NO_2^- in a pH-dependent manner. The findings are consistent with the hypothesis that *N. europaea* expresses NirK as a defence against the toxic NO_2^- that is produced during nitrification.

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Introduction

Nitrosomonas europaea is classically described as a lithoautotrophic bacterium that acquires free energy from the aerobic oxidation of NH_3 to NO_2^- in the process of nitrification (Winogradsky, 1892; Madigan *et al.*, 2000). More recently, the observation of NO and N_2O production via the reduction of NO_2^- suggested that *N. europaea* was also capable of denitrification (Poth and Focht, 1985; Remde and Conrad, 1990). Denitrification is a mode of respiration that allows organisms from the group of heterotrophic denitrifying bacteria to use oxides of nitrogen as alternative electron acceptors when oxygen (O_2) is scarce (Zumft, 1997). The mechanism(s) and physiological significance of denitrification in NH_3-oxidizing bacteria, which also require O_2 for the activation of NH_3, are still unclear (Casciotti and Ward, 2001; Beaumont *et al.*, 2002). The NO and N_2O that are produced by NH_3-oxidizing bacteria in natural ecosystems and industrial wastewater treatment plants are thought to affect the ozone layer and contribute to global warming (Conrad, 1996). Thus, denitrification by NH_3-oxidizing bacteria represents a question that is of both fundamental and ecotechnological relevance.

A complete bacterial denitrification pathway comprises the enzymes nitrate reductase, nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase, which together catalyse the sequential reduction of nitrate (NO_3^-) to dinitrogen via the intermediates NO_2^-, NO and N_2O (Zumft, 1997). Recently, we identified genes encoding NirK and NorCB in *N. europaea* (Beaumont *et al.*, 2002; 2004). Physiological evidence suggests that these bacteria might recruit NirK as protection against NO_2^-, the toxic product of NH_3 oxidation (Poth and Focht, 1985; Stein and Arp, 1998; Beaumont *et al.*, 2002). Alternatively, these enzymes may function as a 'true' denitrification pathway, facilitating free-energy transduction with NO_2^- and NO as terminal electron acceptors, as based on reports of NO_2^--respiration supporting anaerobic growth of *N. europaea* (Abeliovich and Vonshak, 1992; Bock *et al.*, 1995). However, thus far this process has not been experimentally demonstrated to involve NirK.

In virtually all denitrifying bacteria, the concentrations of O_2, NO_3^-, NO_2^- and NO are sensed by multiple transcription regulators that act in concert, giving rise to a response that ensures: (i) full expression of the denitrification enzymes only when the O_2 concentration is low and one of the alternative electron acceptors, NO_3^- or NO_2^-, is present and (ii) maintenance of the toxic intermediates NO_2^- and NO below cytotoxic levels (Zumft, 1997). Reports on the regulation of NirK expression in *N. europaea* are controversial. Miller and Nicholas (1985) described a copper-containing Nir that was induced at low O_2 tensions. However, the authors could not exclude a role of NO_2^- in the observed induction. In contrast, Whittaker *et al*. (2000) reported that the Nir activity in cells that had been grown at high and low O_2 levels was comparable, and hypothesized that the activity of NirK might be regulated at the metabolic level rather than at the level of transcription/translation.

The putative *fnr* gene of *N. europaea*, homologues of which encode transcription regulators that control the expression of the denitrification enzymes in response to low O_2 concentrations in most denitrifying bacteria, was not essential for the expression of NirK or NorCB in *N. europaea* (Beaumont *et al*., 2002; 2004). To date, three regulatory systems that control the expression of bacterial denitrification enzymes in response to NO_2^- have been characterized. NarX-NarL and NarQ-NarP are both two-component regulatory systems that sense NO_3^- and NO_2^- (Stewart and Parales, 1988; Chiang *et al*., 1992). Additionally, NarQ mediates a response to aeration at the transcriptional level (Stewart *et al*., 2003). The third, NarR, is a member of the Fnr family that also senses both NO_3^- and NO_2^- (Wood *et al*., 2001). The activation of NarR by azide (NaN_3), which binds to metal centres, raises the possibility that sensing by NarR involves a metal centre (Wood *et al*., 2002). We did not detect homologues of *narX*, *narL*, *narQ*, *narP* or *narR* in the genome sequence of *N. europaea* (Chain *et al*., 2003).

A more detailed understanding of the regulation of expression of the denitrification enzymes in NH_3-oxidizing bacteria should increase our understanding of their physiological role. In this article, we report the identification of a gene that encodes a novel type of NO_2^--sensing transcription repressor, designated NsrR, which is involved in the regulation of NirK expression in *N. europaea*. We present evidence that identifies the concentration of NO_2^- and the pH as key environmental variables that jointly control the expression of NirK, and illustrates that NsrR is required for this response. Additionally, we discuss the properties of NsrR on the basis of experiments in the heterologous host *Escherichia coli*, which suggest that repression of transcription by NsrR is reversed by NO_2^- in a pH-dependent manner.

Results

Characteristics of NirK expression

Cells of *N. europaea* were grown in batch cultures under lithoautotrophic aerobic conditions and harvested in the early exponential growth phase [optical density at 600 nm (OD_{600}) of 0.02] and in the early stationary growth phase (OD_{600} 0.1). Subsequently, the hydroxylamine-(NH_2OH)-dependent NirK activity was determined in lysates prepared from these cells (Fig. 1). In the stationary growth phase, this NirK activity was approximately sevenfold higher than in the early exponential growth phase.

To allow a more direct and detailed study of the expression profile of NirK, polyclonal antibodies were raised against NirK. The specificity of the obtained antibodies was confirmed by Western blot analysis of lysates of wild-type and NirK-deficient cells, the latter having been described previously (Beaumont *et al*., 2002) (Fig. 2A). Next, lysates were prepared from cells that had been harvested at increasing cell densities from multiple aerobic batch cultures. Measurements with a Clark-type electrode in the early exponential growth phase demonstrated the presence of an excess of O_2 (data not shown). Moreover, aerobic growth could be inferred from the exponential kinetics of growth. Western blot analysis of the specific amount of NirK in these lysates corroborated the upregulation that was detected with the NirK activity measurements (Fig. 2B). The cultures were inoculated with 1.33% (v/v) of a batch culture that was in the early stationary phase of growth. Quantification of the initial decrease of the NirK content revealed that this was inversely proportional to the increase of the cell density, reaching a minimum at an OD_{600} of 0.03. After that point, the amount of NirK increased until a maximum was reached simulta-

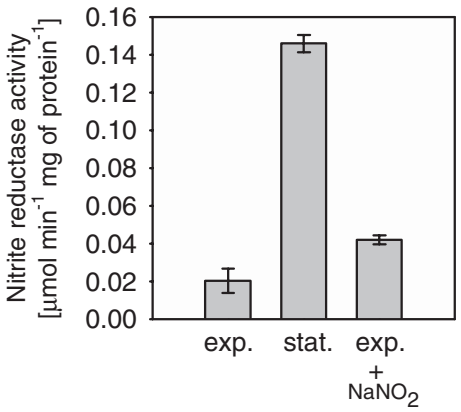


Fig. 1. Specific NH_2OH-dependent NirK activity in lysates of wild-type cells of *N. europaea* that were harvested in the early exponential growth phase at an OD_{600} of 0.02 (exp.); in the early stationary phase at an OD_{600} of 0.1 (stat.); and in the early exponential growth phase at an OD_{600} of 0.02 from cultures to which 15 mM of $NaNO_2$ had been added at the start of culturing (exp. + $NaNO_2$). Error bars indicate the 95% confidence interval of replicate cultures ($n = 2$).

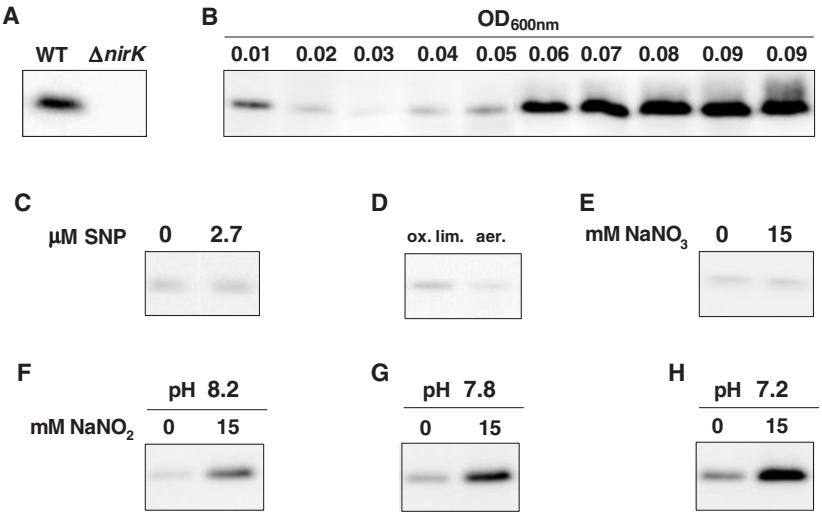


Fig. 2. Western blot detection of NirK in cell lysates of *N. europaea*.
A. NirK in wild-type and NirK-deficient cells that were harvested in the early stationary phase of growth.
B. NirK in wild-type cells that were harvested at increasing cell densities during growth from multiple aerobic batch cultures. The stationary growth phase was reached at an OD_{600} of 0.09; the second 0.09 sample was taken 4 h after the first.
C. NirK in wild-type cells grown to an OD_{600} of 0.02 in the presence of 0 and 2.7 μM of SNP.
D. NirK in wild-type cells grown to an OD_{600} of 0.03 in O_2-limited (ox. lim.) and aerobic cultures (aer.).
E. NirK in wild-type cells grown to an OD_{600} of 0.02 in the presence of 0 and 15 mM of $NaNO_3$.
F–H. NirK in wild-type cells grown to an OD_{600} of 0.02 in the presence of 0 and 15 mM of $NaNO_2$ in cultures that had a starting pH of 8.2, 7.8 and 7.2 respectively. The images are sections of the same Western blot and fully comparable.

neously with the onset of the stationary growth phase (Fig. 2B). The amount of NirK in cells that were in the stationary growth phase was ≈ 18-fold higher than that at an OD_{600} of 0.03 in the early exponential growth phase.

Nitrosomonas europaea *expresses NirK in response to* NO_2^- and pH

In many denitrifying bacteria, Nir is expressed in response to the presence of NO or the lack of O_2 (Zumft, 1997). With the objective to test whether the observed induction of NirK involved NO, cells of *N. europaea* were grown to an OD_{600} of 0.02 in the presence of 0 and 2.7 μM of the NO-releasing agent sodium nitroprusside (SNP), which is commonly used to induce Nir expression in denitrifying bacteria (Kwiatkowski and Shapleigh, 1996) (Fig. 2C). Although the addition of SNP resulted in a reduction of the growth rate, the amount of NirK in cells that had been exposed to SNP was not significantly different from that in cells that had been grown in the absence of SNP.

Next, the effect of O_2 on the expression of NirK was assessed by growing cells to an OD_{600} of 0.03 in aerobic and in O_2-limited batch cultures. O_2 limitation was achieved by shaking the culture flasks at 70 r.p.m., which resulted in linear rather than exponential growth kinetics. Measurements with a Clark-type electrode showed that the O_2 concentration in these flasks was below the detection level of ≈1 μM (data not shown). Western blot analysis revealed that this growth condition had a relatively small, but reproducible, positive effect on the amount of NirK in the cells (Fig. 2D). During incubation, the NO_2^- concentration in the aerobic cultures increased to 4.60 ± 0.07 mM, while the concentration of NO_2^- in the O_2-limited cultures had increased to 5.38 ± 0.05 mM NO_2^-. Additionally, O_2-limited cells had a lower growth rate, reaching the OD_{600} of 0.03 ≈ 12 h later than in aerobic cultures.

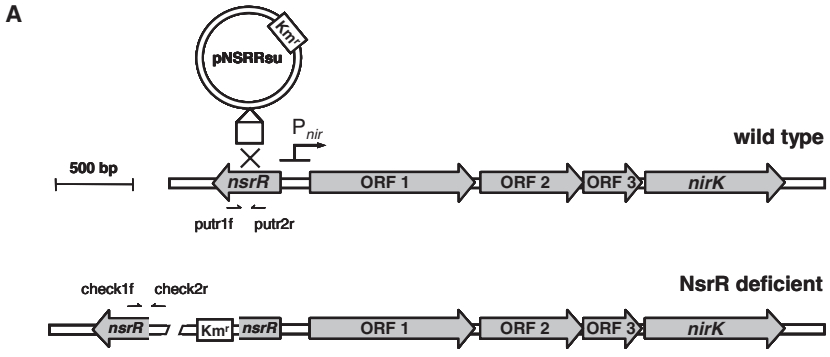
The concentration of NO_2^- in the growth medium typically increased from 0.25 mM at the start of cultivation to 20 mM at the onset of the stationary growth phase. In order to probe whether the concentration of NO_2^- affected the expression level of NirK, the NH_2OH-dependent NirK activity was determined in lysates of cells that had been harvested at an OD_{600} of 0.02 from cultures to which 15 mM $NaNO_2$ was added at the start of culturing (Fig. 1). Cells in these cultures expressed a level of NirK activity that was approximately twofold higher than in cultures to which no $NaNO_2$ had been added. This finding was corroborated by Western blot analysis of NirK in cells from a similar experiment, which revealed that the addition of 15 mM $NaNO_2$ resulted in an approximately twofold increase of the NirK content of the cells (Fig. 2F). In a control experiment, 15 mM $NaNO_3$ was added instead of $NaNO_2$, which did not significantly affect the expression of NirK (Fig. 2E).

During growth in batch cultures, the pH of the growth medium typically decreased from 8 to 5.7 as a result of the conversion of NH_3 to NO_2^- and protons (H^+). The effect of the extracellular pH on the expression of NirK was assessed by growing cells to an OD_{600} of 0.02 in medium of which the pH was set to 8.2, 7.8 and 7.2 respectively (Fig. 2F–H). This established that the expression level of NirK increased with decreasing pH. The pH of the cultures with initial pH 8.2 and 7.8 both decreased to 7.6, while the pH of the culture with starting pH 7.2 did not change during culturing to an OD_{600} of 0.02. These differences

probably resulted from the fact that the phosphate used as pH buffer has a pK_{a2} of 7.2.

The nsrR *gene of* N. europaea

The *nirK* gene is clustered with three other open reading frames (ORFs) in the genome of *N. europaea* (Beaumont *et al*., 2002; Chain *et al*., 2003) (Fig. 3A). ORF 1 encodes a periplasmic copper-containing protein with homology to members of the family of copper oxidases, which was shown to possess oxidase activity and a minor nitrite reductase activity (DiSpirito *et al*., 1985; Beaumont *et al*., 2002). ORFs 2 and 3 encode *c*-type haem containing proteins of which the function is unknown (Whittaker *et al*., 2000). At present, this *nirK* gene cluster organization is unique to *N. europaea*; in the heterotrophic denitrifying bacteria characterized so far, *nirK* is in some cases clustered with *nirV*, which encodes an enzyme of unknown function (Jain and Shapleigh, 2001). The *nirK* gene cluster is preceded by an ORF that has homology to genes encoding members of the Rrf2 family of putative transcription regulators (Pfam PF02082, see http://pfam.wustl.edu/cgi-bin/getdesc?name=Rrf2 for species distribution and alignment; Bateman *et al*., 2004). We have designated this gene *nsrR* (nitrite-sensitive repressor). In *Desulfovibrio vulgaris*, inactivation of the *rrf*2 gene resulted in the overexpression of the *hmc* operon, which encodes a redox protein that is involved in electron transport during hydrogen oxidation with sulphate as electron acceptor (Keon *et al*., 1997). RirA of *Rhizobium leguminosarum*, a second Rrf2 family member that was experimentally shown to be involved in transcription regulation, was crucial for gene expression in response to iron and has been proposed to be an iron-responsive regulator of transcription (Todd *et al*., 2002). To date, no other members of this family have been characterized. The helix–turn–helix (HTH) algorithm of Dodd and Egan predicted the presence of an HTH DNA-binding domain with a likelihood of 90% in Rrf2 and NsrR, and 50% in RirA (Fig. 3B) (Dodd and Egan, 1990; Combet *et al*., 2000). Overall, this algorithm predicted the presence of an HTH motif with a likelihood of 25% or higher for 77 of the 180 Rrf2 family members in the current Pfam database. NsrR contained five cysteine residues, three of which appeared to be conserved in ≈ 70% of the Rrf2 family members (Fig. 3B). The presence of these cysteines did not correlate with the presence of a predicted HTH motif. A search of the COG database using the STRING tool for retrieval of interacting genes/proteins (COG1959; Tatusov *et al*., 2003) (http://dag.embl-heidelberg.de:8080/newstring_cgi/



B

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      1                                                  50
NsrR  MRLTNYSDYA LRILTYLGLK REELS.TITE IADCYGISRN HVVKIVHHLG
RirA  MRLTKQTNYA VRMLMYCAAN DGHLS.RIPE IAKAYGVSEL FLFKILQPLN
Rrf2  MKLTTRSRYG TRMLLDIAMH GSEQPVSIKD IAQRQGISVK YLEKLIRVLR

      51                                                 100
NsrR  QLGYVDTLRG KNGGIRLAHA PEKINIGEVI RHTETSMDIV ECFSNQNS..
RirA  KAGLVETVRG RNGGVRLGKP AADISLFDVV RVTEDSFAMA ECFEDDGEVE
Rrf2  KAGYITSTLG AHGGYQLAHK AEDIPVGDVV YALEEMEAPV ECDEENPC..
                                                       *
      101                                                150
NsrR  CIIGCSCVLR TAISEALSAF MAVLDDYTLA DLIAPRRQLS RKLHVMQISD
RirA  CPLVDSCGLN SALRKALNAF FAVLSEYSID DLVKARPQIN FLLGITGEPA
Rrf2  CPRMAVCLTR TIWGEAAKAM YRKLNTFTLA DLMRDASLCP KNACNISPHA
      *     *
      151      161
NsrR  SLSD..... .
RirA  YRKPAIVAPA A
Rrf2  TH........ .
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Fig. 3. Genomic context and mutagenesis of the *nsrR* gene, and comparison of the primary sequences of the characterized Rrf2-type transcription regulators.
A. Schematic representation of the *nirK* gene cluster and the *nsrR* gene (NE0928) in the wild-type and in the NsrR-deficient strains of *N. europaea*. Small arrows indicate the primers used for the construction of the suicide vector and verification of the mutant organization.
B. Alignment of the amino acid sequences of NsrR of *N. europaea* (NCBI Accession No. NP841002), Rrf2 of *Desulfovibrio vulgaris* (NCBI Accession No. P33395) and RirA of *Rhizobium leguminosarum* (NCBI Accession No. CAC35510) that was generated with Multalin (Corpet, 1988). Underlined characters indicate the predicted helix–turn–helix motif, shaded characters indicate conserved residues and astrixes indicate conserved cysteine residues.

show_input_page.pl) revealed that in 21 of the 61 species that contained one or more *rrf2* homologue, at least one of these genes was located in the vicinity of genes that appeared to encode enzymes involved in the biosynthesis of iron–sulphur clusters.

The nsrR *gene is involved in the regulation of NirK expression*

The possible involvement of the *nsrR* gene in the regulation of NirK expression was assessed by the inactivation of *nsrR* of *N. europaea* through the insertion of a suicide vector via homologous recombination (Fig. 3A). Correct integration of the suicide vector was confirmed by polymerase chain reaction (PCR) (data not shown). In the early exponential growth phase (OD_{600} 0.02), NsrR-deficient cells expressed NirK at a level that was ≈ 40-fold higher than that observed in wild-type cells in the same growth phase (Fig. 4A). In the early stationary growth phase (OD_{600} 0.06), the amount of NirK in NsrR-deficient cells was approximately twofold higher than in wild-type cells (Fig. 4A). Additionally, disruption of the *nsrR* gene reduced both the growth rate and the maximal cell density that was reached in batch cultures (Fig. 4B).

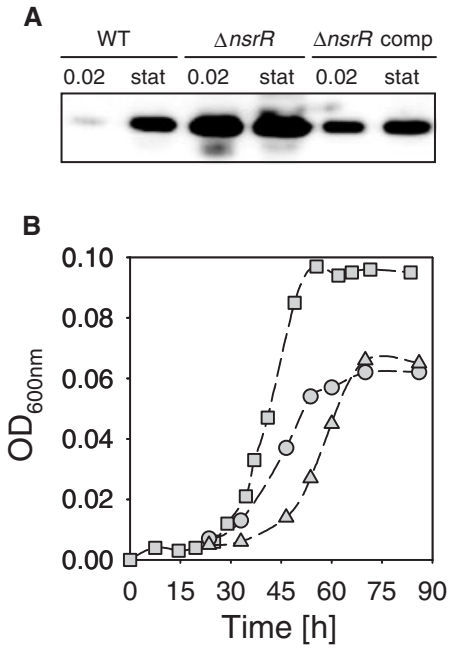

Fig. 4. The effects of inactivation of *nsrR* on NirK expression and growth.
A. Western blot detection of NirK in lysates from wild-type (WT), NsrR-deficient (Δ*nsrR*) and complemented NsrR-deficient cells (Δ*nsrR*comp), which were harvested at an OD_{600} of 0.02 in the early exponential phase and in the early stationary phase (stat).
B. Growth curves of wild-type (squares), NsrR-deficient (circles) and complemented NsrR-deficient cells (triangles) in aerobic batch cultures.

As a control for secondary effects of the disruption of the *nsrR* locus, a complementation vector that harboured an intact copy of the *nsrR* gene under the control of its native promoter was inserted in NsrR-deficient cells. This did not redress the negative effects of the inactivation of *nsrR* on growth, but did lower the level of NirK expression in the early stationary growth phase to that observed in the wild-type (Fig. 4A and B). In the early exponential growth phase, the amount of NirK in the NsrR-deficient cells that carried the complementation vector was still ≈ 20-fold higher than in wild-type cells.

NsrR controls the activity of the nirK *gene cluster promoter*

To establish whether the region upstream of ORF 1 encodes a functional promoter and to measure the activity of this putative *nirK* gene cluster promoter (P_{nir}) in cells during growth in batch cultures, the intergenic region between *nsrR* and ORF 1 was cloned in vector pMP190 (Spaink *et al*., 1987) upstream of a promoterless *lacZ* gene (Fig. 3A). This P_{nir} reporter vector was transferred to wild-type and NsrR-deficient cells of *N. europaea*. Both strains were grown in aerobic batch cultures that were inoculated with 1.33% (v/v) of a preculture that was in the early stationary growth phase. Cells were harvested from single cultures at increasing cell densities for the determination of the specific β-galactosidase activity, which was used as a measure for the activity of P_{nir}. In wild-type cells, the activity of P_{nir} increased approximately linearly with increasing cell density, from 1000 Miller units in the early exponential growth phase to ≈ 4500 Miller units at the onset of the stationary growth phase (Fig. 5A). In contrast, NsrR-deficient cells expressed β-galactosidase activity at a more constant level that varied between ≈ 2000 and 3000 Miller units in a manner that did not correlate with the increasing cell density (Fig. 5B). The amount of NirK in these cells was approximately the same as in cells that did not harbour the reporter vector, demonstrating that the presence of the P_{nir} reporter vector had no effect on the expression level of NirK (data not shown).

NsrR represses transcription from P_{nir} *in* E. coli

To investigate whether NsrR was also able to repress transcription from the *nirK* gene cluster promoter in a heterologous background, the P_{nir} reporter vector was inserted in *E. coli* TG1 (*lacZ*$^-$). These cells expressed β-galactosidase activity, demonstrating that P_{nir} was a functional promoter in *E. coli* (Fig. 6A). Next, an NsrR expression vector (pNSRR) was inserted in these P_{nir} reporter vector harbouring cells. This resulted in a 15-fold lower expression level of β-galactosidase from the P_{nir} reporter vector (Fig. 6A). A control vector, which contained another

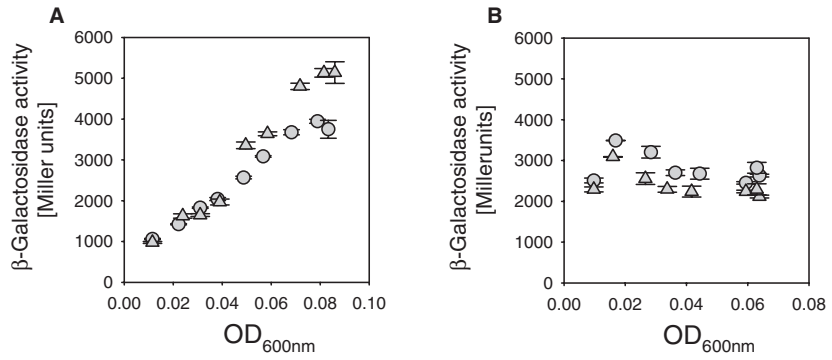

Fig. 5. Specific β-galactosidase activities in wild-type (A) and NsrR-deficient (B) cells of *N. europaea* that contained the P_{nir} reporter vector during growth in two replicate batch cultures (triangles and circles). Error bars indicate the 95% confidence interval of the β-galactosidase activity measurement ($n = 3$).

DNA fragment than the *nsrR* gene, had only a slight effect on the level of β-galactosidase expressed from the P_{nir} reporter construct (Fig. 6A).

NO_2^- reverses repression by NsrR in E. coli *in a pH-dependent manner*

To study the effects of NO_2^- on transcription repression by NsrR, increasing amounts of $NaNO_2$ were added to cultures of exponentially growing cells of *E. coli* that harboured both the P_{nir} reporter vector and the NsrR expression vector. β-Galactosidase activities were determined after 1 h of exposure to 0, 20, 40 and 60 mM of $NaNO_2$ respectively (Fig. 6B, grey bars). The β-galactosidase activity increased approximately linearly with the concentration of $NaNO_2$. Because *E. coli* contains two genes, *yfhP* and *yjeB*, that are predicted to encode proteins that belong to the Rrf2 family, and in order to verify the dependence of the observed phenomenon on NsrR, the effects of NO_2^- on the activity of P_{nir} were also studied in an *E. coli* strain that harboured only the P_{nir} reporter vector. The β-galactosidase activity in these cells was not significantly affected by exposure to these amounts of $NaNO_2$ (data not shown). Exposure to a 10-fold lower range of $NaNO_2$ concentrations (i.e. 0, 2, 4 and 6 mM) at a pH that was one unit lower than in the first experiment (i.e. pH 5.3 instead of 6.3) resulted in approximately the same expression levels of β-galactosidase activity from the P_{nir} reporter construct (Fig. 6B, white bars).

With the aim to assess the effect of NO_2^- on transcription repression by NsrR without the sudden confrontation of the cells to externally added NO_2^-, cells of *E. coli* that harboured both the P_{nir} reporter vector and the *nsrR* expression vector were cultured under O_2-limiting conditions with nitrate (NO_3^-) as alternative electron acceptor. The rationale behind this approach was to introduce NO_2^-, the reduction product of NO_3^-, in the cytoplasm by the action of membrane bound nitrate reductase during O_2-

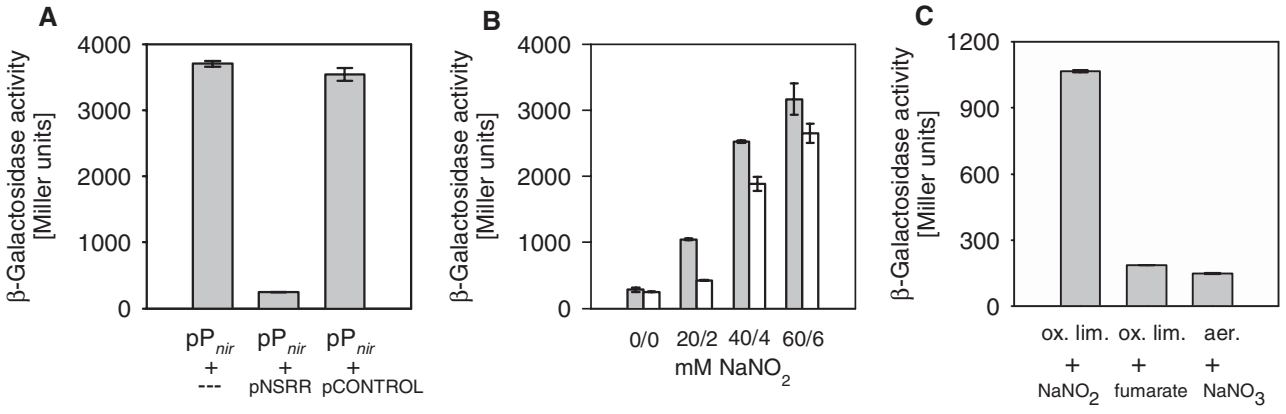

Fig. 6. Characteristics of repression of transcription from P_{nir} by NsrR in *E. coli*.
A. Specific β-galactosidase activities in cells of *E. coli* harbouring the P_{nir} reporter vector (pP_{nir}); the P_{nir} reporter vector and the NsrR expression vector (pP_{nir} + pNSRR); and the P_{nir} reporter vector and a control vector (pP_{nir} + pCONTROL). Error bars indicate the 95% confidence interval of the β-galactosidase activity measurement ($n = 3$).
B. Specific β-galactosidase activities in cells of *E. coli* that harboured both the P_{nir} reporter vector and the NsrR expression vector that were exposed for 1 h to 0, 20, 40 and 60 mM of $NaNO_2$ at pH 6.3 (grey bars), and 0, 2, 4 and 6 mM of $NaNO_2$ at pH 5.3 (white bars). Error bars indicate the 95% confidence interval of the β-galactosidase activity measurement ($n = 3$).
C. Specific β-galactosidase activities in cells of *E. coli* that harboured pP_{nir} and pNsrR in the presence of 50 mM $NaNO_3$ or 30 mM fumarate, under aerobic (aer.) or O_2-limiting (ox. lim.) conditions. Error bars indicate the 95% confidence interval of the β-galactosidase activity measurement ($n = 3$).

limited denitrifying growth (Zumft, 1997). The cells were cultured in the presence of 50 mM of $NaNO_3$ under aerobic and under O_2-limiting conditions. In a control experiment, fumarate was added as alternative electron acceptor. Cells that had been cultured overnight in the presence of NO_3^- under O_2 limitation exhibited a β-galactosidase activity that was approximately fivefold higher than that observed after aerobic growth with NO_3^-, or after O_2-limited growth in the presence of fumarate (Fig. 6C). The β-galactosidase activity in cells that harboured only the P_{nir} reporter construct was not significantly differentially affected by these growth conditions (data not shown).

Discussion

We have identified a gene that encodes a novel NO_2^--sensing transcription repressor in *N. europaea*, designated NsrR, which plays a crucial role in the expression of NirK in response to the concentration of NO_2^- and the pH. NsrR of *N. europaea* is a member of the Rrf2 family of putative transcription regulators, which appear to be wide spread among the *Bacteria*. So far, the only Rrf2 family member for which a signal has been suggested is the iron-sensing RirA of *R. leguminosarum* (Todd *et al*., 2002).

We propose that the concentration of NO_2^- and the pH, and not NO or O_2, are the main environmental variables that control the expression of NirK in *N. europaea* on the basis of the following observations: (i) aerobically growing cells of *N. europaea* expressed increasing amounts of NirK in response to the addition of NO_2^- and lowering of the pH of the growth medium, (ii) the addition of the NO-releasing agent SNP had no significant effect on the NirK content of cells and (iii) O_2-limiting growth conditions had a relatively small inducing effect. However, in the later experiment, the amount of NO_2^- produced during growth and the growth rate also varied as a result of the O_2-limiting growth conditions, hampering the unambiguous inference of the specific role of O_2 in this response. Nevertheless, considering the magnitudes of the effects of NO_2^- and pH, compared to that of O_2-limitation, it is clear that if O_2 affects the expression of NirK under these conditions at all, this merely involves the modulation around a level that is dictated by the concentration of NO_2^- and the pH.

The finding that the amount of NirK in cells decreased linearly with the increase of the cell density during the early exponential growth phase is consistent with distribution of NirK over daughter cells in the absence of *de novo* NirK synthesis, indicating that under these conditions NirK synthesis is in the off state.

The involvement of the *nsrR* gene in the regulation of NirK expression was demonstrated by experiments with an NsrR-deficient mutant. In this strain, NirK was constitutively expressed regardless of the cell density. The inactivation of *nsrR* was partially complemented by the introduction of an intact *nsrR* gene under the control of its native promoter *in trans*, which reduced the overall expression level of NirK, but did not revert the constitutive expression to the expression pattern observed in wild-type cells. Presumably, this reflected the sensitivity of the regulation of NirK expression by NsrR to changes in the *nsrR* gene copy number or genetic context (i.e. genomic versus vector borne). Alternatively, this may signify the presence of additional regulatory mechanisms that are involved the transcription of *nirK*.

NsrR repressed transcription from P_{nir}, a promoter upstream of the *nirK* gene cluster that was hypothesized to be targeted by NsrR. The activity of P_{nir} in wild-type and NsrR-deficient cells of *N. europaea* resembled the expression profile of NirK qualitatively, as measured with a P_{nir}-*lacZ* fusion present *in trans*. However, the maximal level of β-galactosidase activity in NsrR-deficient cells was lower than that observed in a wild-type background (Fig. 5A). This was in contrast with NirK, which was expressed in the NsrR-deficient strain at a level that was twofold higher than in wild-type cells (Fig. 4A). This discrepancy did not result from the presence of the reporter construct because the level of NirK expression was not affected by the presence of this construct. These findings raise the possibility that the transcription of NirK is not (solely) under the direct control of P_{nir}. Conversely, the activity of P_{nir} may be dependent on its genetic context.

On the bases of experiments in which the NsrR-P_{nir} system was reconstructed in the heterologous host *E. coli*, we conclude that repression of transcription from P_{nir} by NsrR is reversed by NO_2^- in a manner that depended on the extracellular pH. Lowering of the extracellular pH in the absence of NO_2^- did not significantly affect repression. In contrast, in the presence of NO_2^-, the lowering of the pH by 1 unit resulted in an ≈ 10-fold increase of the sensitivity of NsrR to NO_2^-. We propose that the effect of pH on transcription repression by NsrR is not direct, but rather acts via modulation of the rate at which NO_2^- enters the cytoplasm. Studies in other bacteria have illustrated that this rate depends on the extracellular pH, which, when decreased, shifts the equilibrium between NO_2^- and HNO_2 toward the protonated form that passes through the cytoplasmic membrane at higher rates than the anionic form (Maeda *et al*., 1998; Wu and Stewart, 1998). At this moment, it remains unclear whether NsrR responds to NO_2^- or HNO_2. Unlike NarR, which is activated by NaN_3 (Wood *et al*., 2002), repression by NsrR in *E. coli* was not sensitive to NaN_3 (data not shown).

Taken together, the evidence presented here suggests that the expression of NirK in *N. europaea* is regulated through NsrR, which represses transcription from the pro-

moter P_{nir}. Repression by NsrR is reversed by intracellular NO_2^-, or HNO_2, and thereby induction of transcription from P_{nir} increases with increasing extracellular concentrations of NO_2^- and with decreasing extracellular pH. The possibility that additional regulatory mechanisms are involved in the regulation of NirK expression cannot be ruled out at present. Clearly, as suggested previously by Whittaker *et al*., the activity of NirK in *N. europaea* must also be regulated at the post-translational or metabolic level. Cells of *N. europaea* contain a potential specific Nir activity that is comparable to that of the bacterium *Paracoccus denitrificans* during denitrifying growth, but nonetheless reduce no more than 5% of the NO_2^- produced during O_2-limited growth (Hynes and Knowles, 1984; Anderson *et al*., 1993; Kester *et al*., 1997; Jiang and Bakken, 1999; Saunders *et al*., 1999; Whittaker *et al*., 2000). Unlike Nir in most denitrifying bacteria, NirK of *N.* europaea is expressed under fully aerobic conditions in response to NO_2^-. This finding is in agreement with the previously formulated hypothesis that *N. europaea* recruits NirK during ammonia oxidation to counteract the toxic effects of NO_2^- (Poth and Focht, 1985; Beaumont *et al*., 2002).

Experimental procedures

Bacterial strains, media and growth conditions

Nitrosomonas europaea [strains ATCC 19718 and the derived NsrR-deficient strain TLnt (*nsrR*::pNSRRsu, Km^r, described in this work)] was cultured in liquid mineral *Nitrosomonas* medium (pH 8) that contained 25 mM $(NH_4)_2SO_4$ as free energy and nitrogen source at 30°C in the dark as described by Hyman and Arp (1992). Growth curves of *N. europaea* were determined in 150 ml batch cultures in 500 ml flasks that were inoculated with a 0.8% (v/v) inoculum of an early stationary growth phase culture of OD_{600} of 0.1, and incubated with loose caps for gas exchange on an angled (70°) rotary shaker (175 r.p.m.) at 30°C in the dark. Cells of *N. europaea* for the quantification of NirK by activity measurements and Western blot analyses were cultured in 2 l Erlenmeyer flasks containing 1.5 l of liquid *Nitrosomonas* mineral medium that was inoculated with a 1.33% (v/v) inoculum of an early stationary growth phase culture of OD_{600} of 0.1, and incubated on a rotary shaker (175 r.p.m.) at 30°C in the dark. O_2-limited growth of cells of *N. europaea* was achieved by culturing in slowly shaken (70 r.p.m.) 1.5 l batch cultures in 2 l Erlenmeyers. Culturing on nylon filters placed on solid medium at 30°C was performed as described previously (Hommes *et al*., 1996). *E. coli* strains TG1 (Sambrook *et al*., 1989), S17-1 (Sm^r; Simon *et al*., 1983) and HB101 (Boyer and Roulland-Dussoix, 1969) were cultured at 37°C (200 r.p.m.) in yeast-tryptone (YT) medium (Ausubel *et al*., 1983). For the determination of the effects of NO_2^-, NO_3^-, SNP and sodium azide on the expression of NirK, $NaNO_2$, $NaNO_3$, $Na_2[Fe(CN)_5NO]\cdot H_2O$ and NaN_3, respectively, were added to the media for *N. europaea* or *E. coli* from filter-sterilized stock solutions. For determination of the effect of the initial pH of the growth medium on the expression of NirK, liquid mineral *Nitrosomonas* medium of the desired pH was prepared by the addition of less NaOH to the medium. Kanamycin (25 μg ml^{-1}), streptomycin (25 μg ml^{-1}), tetracyclin (6 μg ml^{-1}), nalidixic acid (30 μg ml^{-1}) or combinations of these antibiotics were added to the media for the selection and maintenance of mutant or plasmid harbouring strains of *N. europaea* and *E. coli* when necessary. Cell densities were determined by measurement of the OD_{600} with a spectrophotometer. NO_2^- in the growth medium was measured colorimetrically as described previously (Nicholas and Nason, 1957).

Determination of NirK activity

The NirK activity was determined using hydroxylamine (NH_2OH) as electron donor in cell lysates as described previously (Hooper, 1968). Because this assay depends on electrons released by the *N. europaea* enzyme hydroxylamine oxidoreductase (HAO), changes in the rate of NO_2^- consumption may reflect both changes in the expression of NirK and/or HAO. Lysates were made by sonication of washed cells (50 mM Tris-HCl, pH 8) that had been harvested at the appropriate cell density. Cell debris was removed by centrifugation (5 min, 10.000 *g*). Protein concentrations were determined with the BCA protein assay kit (Pierce). The assay was performed in 1 ml 50 mM NaAc (pH 5.75) containing 1 mM $NaNO_2$, 100 mM $NH_2OH\cdot HCl$ and 50 μg ml^{-1} protein at 25°C in open reaction vials. The disappearance of NO_2^- from the reaction mixture was measured colorimetrically as described previously (Nicholas and Nason, 1957).

Western blot detection of NirK

Rabbit serum containing polyclonal antibodies against NirK was produced by immunization with two synthetic NirK oligopeptides based on the *N. europaea nirK* gene sequence (NE0924, amino acid residues 93–107 and 295–309), coupled to bovine serum albumin as a carrier protein by AgriSera AB (Sweden). Lysates of *N. europaea* cells were prepared in phosphate buffer (40 mM KH_2PO_4, 3.5 mM NaH_2PO_4, adjusted with NaOH to pH 8.0) by the same method as described above for the determination of NirK activity. For Western blot detection of NirK, equal amounts of lysate (15 μg protein per lane) were separated by SDS-PAGE (12% gel), followed by transferal of the separated proteins to PVDF membranes (Immun-Blot, Bio-Rad) by overnight electroblotting at 4°C. Next, the PVDF membranes were incubated with an excess of the NirK antibodies containing serum, and subsequently with a goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad). Signal was generated using the Lumi-Light^{plus} Western blotting substrate (Roche) and subsequently detected and quantified with the Bio-Rad Imager system and software (Bio-Rad). Signals were in the linear range of the detection method.

Construction of the NsrR-deficient mutant

The *nsrR* gene was disrupted by insertion of suicide vector pNSRRsu via homologous recombination involving a single

cross-over event (Fig. 3). For the construction of suicide vector pNSRRsu, a 167 bp internal fragment of the *nsrR* gene was amplified by PCR with primers putr1f (5′-CGG TATTTCGCGCAAC-3′) and putr2r (5′-CGACGATATCCATCG AAG-3′) and subcloned in vector pGEMT-easy (Promega). Subsequently, the fragment was excised from pGEMT-easy with *Eco*RI and inserted into vector pRVS3 (Km^r; van Spanning *et al*., 1995) that is replicated in *E. coli* but not in *N. europaea*, creating suicide vector pNSRRsu, which was transferred to *N. europaea* via single mating conjugation as described below. Integration of pNSRRsu in the targeted locus was verified by PCR with one gene-specific (check1f) and one vector-specific primer (check2r), which only yields a product with the mutant organization (Fig. 3).

Construction of pNSRRco for complementation study

For the construction of complementation vector pNSRRco, a 681 bp fragment encompassing the *nsrR* gene and its native promoter region (195 bp upstream of *nsrR*) was amplified by PCR with primers putr3f (5′-CTGCACGAAAGACCTTTA AATAC-3′) and putr4r (5′-CGGTCGATCTGTTCTACAAC-3′), subcloned in vector pGEMT-easy, excised with *Sal*I and *Sph*I, and subcloned in vector pGEM3 (Promega). Subsequently, the fragment was excised with *Bam*HI and *Hin*dIII and inserted into the broad-host range vector pEG400 (Sm^r, IncP; Gerhus *et al*., 1990), creating complementation vector pNSR-Rco, which was transferred to *N. europaea* via triple mating as described below.

Construction of pP_{nir} for promoter activity study

For the determination of the activity of the *nirK* gene cluster promoter P_{nir}, a DNA fragment that extended 117 bp into *nsrR* and 7 bp into ORF 1, encompassing the 188 bp intergenic region between *nsrR* and ORF 1 designated P_{nir}, was amplified by PCR with primers pcop1f (5′-GTTGCGCGAA ATACCG-3′) and putr3f. Subsequently, this fragment was subcloned in vector pGEMT-easy, excised with *Eco*RI, and subcloned into vector pGEM7 (Promega). Next, the fragment was excised from pGEM7 with *Xba*I and *Bam*HI and inserted into *Xba*I–*Bgl*II digested pMP190 (Sm^r; Spaink *et al*., 1987) upstream of the promoterless *lacZ* gene, creating the P_{nir}-*lacZ* fusion reporter vector pP_{nir}. This vector was transferred to wild-type and NsrR-deficient cells of *N. europaea* via triple mating as described below.

Construction of pNSRR for expression of NsrR in E. coli

For the study of NsrR in *E. coli*, a 551 bp fragment containing the *nsrR* gene was amplified by PCR with primers prexp5f (5′-GCCAAGCTGACCTACAGTTTATTCC-3′) and prexp6r (5′-taaggaggAATATAATATGAGACTGACGAATTACAGCGAT TAC-3′, lower case indicates an inserted Shine-Delgarno sequence for binding of the ribosome, facilitating translation). This fragment was inserted in vector pGEMT-easy and correct orientation of the *nsrR* gene relative to the *lacZ* promoter of pGEMT-easy was confirmed by digestion with *Apa*I and *Sma*I.

Transfer of plasmids from E. coli *to* N. europaea *via conjugation*

Recipient cells of *N. europaea* were harvested in the early stationary growth phase and washed three times with ice-cold sterile phosphate buffer (40 mM KH_2PO_4, 3.5 mM NaH_2PO_4, adjusted with NaOH to pH 8.0), resuspended to an OD_{600} of 8.0 and stored on ice. Donor cells (*E. coli* S17-1 or TG1 harbouring the plasmid to be transferred to *N. europaea*) and helper cells (*E. coli* HB101 harbouring mobility vector pRK2020, Tc^r; Ditta *et al*., 1980) were grown overnight, washed three times in ice-cold phosphate buffer, resuspended in phosphate buffer to an OD_{660nm} of 3.0 and stored on ice. Double mating was performed by mixing 40 μl of *N. europaea* cell suspension with 20 μl of the cell suspension of *E. coli* S17-1. Triple mating was performed by mixing 40 μl of *N. europaea* cell suspension with 20 μl *E. coli* TG1 cell suspension and 20 μl *E. coli* HB101 cell suspension. These cell mixtures were spread (≈ 3 cm^2) on nylon filters (Hybond-N, Amersham Biosciences) that resided on conjugation plates, which contained 5 g l^{-1} tryptone, 2.5 g l^{-1} yeast extract, 9.5 mM $(NH_4)_2SO_4$, 8.0 mM KH_2PO_4, 42.3 mM Na_2HPO_4, 22.2 mM $Mg_2HPO_4{\cdot}7H_2O$, 34.0 μM $CaCl_2{\cdot}2H_2O$, 10.0 μM $FeCl_3$, 13.4 μM EDTA and 15 g l^{-1} Gellan gum (Sigma). This solid conjugation medium was prepared as follows. For 500 ml, appropriate amounts of tryptone, yeast extract, KH_2PO_4 and Na_2HPO_4 were completely dissolved in 250 ml H_2O, after which the Gellan gum was added. This mixture was then autoclaved. Simultaneously, a bottle with 225 ml H_2O was autoclaved after which the appropriate amounts of Mg_2HPO_4, $CaCl_2{\cdot}2H_2O$ $(NH_4)_2SO_4$ and Fe-EDTA were added from concentrated sterile stock solutions. The contents of the two flasks were mixed immediately after autoclaving and distributed over sterile Petri dishes. After application of the filters and spreading of the cell mixtures, conjugation plates were sealed with parafilm (Pechiney Plastic Packaging) and incubated for 3 days at 30°C in the dark.

After conjugation, the filters with the cells were transferred from the conjugation plate to 100 ml liquid mineral *N. europaea* growth medium in a 500 ml bottle. Selection of cells of *N. europaea* in which pNSRRsu was integrated in the chromosome was achieved by the addition of 6 μg ml^{-1} tetracyclin (elimination of the donor *E. coli* S17-1 cells) and 25 μg ml^{-1} kanamycin (elimination of wild-type *N. europaea* cells). Selection of pNSRRco harbouring NsrR-deficient cells of *N. europaea* was achieved by the addition of 30 μg ml^{-1} nalidixic acid (elimination of the donor *E. coli* TG1 and helper *E. coli* HB101 cells), 25 μg ml^{-1} streptomycin (elimination of NsrR-deficient cells of *N. europaea* that did not harbour pNSRRco) and 25 μg ml^{-1} kanamycin (selection for the disrupted *nsrR* gene organization of NsrR-deficient cells). Cells of *N. europaea* harbouring pP_{nir} were selected with of 30 μg ml^{-1} nalidixic acid and 25 μg ml^{-1} streptomycin for wild-type cells and additionally 25 μg ml^{-1} kanamycin for NsrR-deficient cells. These cultures were incubated at 30°C (175 r.p.m.) in the dark until NO_2^- had accumulated to a concentration of ≈ 2 mM [monitored with NO_2^- strips (Merck)], which required 14–20 days of incubation. At this point, 10-fold dilutions (10^0–10^{-5}) were prepared, of which aliquots of 100 μl were spread on nylon filters that were positioned on solid *N. europaea* growth medium and incubated until colonies appeared after ≈ 14 days. The same antibiotics that were used in the liquid

selection medium were used in these plates. Colonies were selected and transferred to a 20 ml tube with 5 ml of liquid mineral *N. europaea* medium containing 25 μg ml^{-1} kanamycin for NsrR-deficient cells of *N. europaea*; 25 μg ml^{-1} streptomycin for wild-type cells harbouring pP_{nir}; and 25 μg ml^{-1} kanamycin and 25 μg ml^{-1} streptomycin for NsrR-deficient cells that harboured pNSRRco or pP_{nir}.

β-Galactosidase activity in N. europaea *and* E. coli

Nitrosomonas europaea cells for the determination of the specific β-galactosidase activity were collected from single 1.5 l batch cultures at increasing cell densities by centrifugation of aliquots (15 min 9000 r.p.m. at 4°C), resuspended in phosphate buffer (40 mM KH_2PO_4, 3.5 mM NaH_2PO_4, adjusted with NaOH to pH 8.0) to an OD_{600} of ≈ 2 and stored at –21°C until further analysis.

For the determination of β-galactosidase activity in exponentially growing pP_{nir} and pNSRR harbouring *E. coli* TG1 after a 1 h exposure to various compounds at pH 6.3 or 5.3, cells were precultured in 75 ml YT medium in a 500 ml bottle until an OD_{600} of ≈ 1 was reached. Subsequently, 5 ml aliquots of this preculture were transferred to 20 ml tubes to which the compounds to be tested were added from filter-sterilized stock solutions, and incubated for 1 h at 37°C (200 r.p.m.). The pH of the cultures before the 1 h incubation was 6.3; 2.8 μl 6 M HCl was added for induction at pH 5.3. After incubation and determination of the OD_{600}, the cultures were stored at –21°C until further analysis.

For the determination of β-galactosidase activity in pP_{nir} and pNSRR harbouring *E. coli* with $NaNO_3$ or fumarate as alternative electron acceptor, cells were cultured statically overnight at 37°C in 20 ml tubes completely filled with YT medium for O_2-limiting conditions, and at 200 r.p.m. overnight in 20 ml tubes with 5 ml YT medium for aerobic cultures, in the presence of either 50 mM $NaNO_3$ or 30 mM fumarate. After determination of the OD_{600}, the cultures were stored at –21°C until further analysis.

Specific β-galactosidase activities in toluene-permeated cells of *N. europaea* and *E. coli* were determined at 28°C as described previously (Miller, 1972), and expressed in arbitrary specific Miller units.

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References

Abeliovich, A., and Vonshak, A. (1992) Anaerobic metabolism of *Nitrosomonas europaea*. *Arch Microbiol* **158:** 267–270.

Anderson, I.C., Poth, M., Homstead, J., and Burdige, D. (1993) A comparison of NO and N_2O production by the autotrophic nitrifier *Nitrosomonas europaea* and the heterotrophic nitrifier *Alcaligenes faecalis*. *Appl Environ Microbiol* **59:** 3525–3533.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1983) *Current Protocols in Molecular Biology*. New York, NY: John Wiley & Sons.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., *et al.* (2004) The Pfam protein families database. *Nucleic Acids Res* **32:** D138–141.

Beaumont, H.J., Hommes, N.G., Sayavedra-Soto, L.A., Arp, D.J., Arciero, D.M., Hooper, A.B., *et al.* (2002) Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. *J Bacteriol* **184:** 2557–2560.

Beaumont, H.J., van Schooten, B., Lens, S.I., Westerhoff, H.V., and van Spanning, R.J. (2004) *Nitrosomonas europaea* expresses a nitric oxide reductase during nitrification. *J Bacteriol*, **186:** 4417–4421.

Bock, E., Schmidt, I., Stüven, R., and Zart, D. (1995) Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. *Arch Microbiol* **163:** 16–20.

Boyer, H.W., and Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41:** 459–472.

Casciotti, K.L., and Ward, B.B. (2001) Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. *Appl Environ Microbiol* **67:** 2213–2221.

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., *et al.* (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J Bacteriol* **185:** 2759–2773.

Chiang, R.C., Cavicchioli, R., and Gunsalus, R.P. (1992) Identification and characterization of *narQ*, a second nitrate sensor for nitrate-dependent gene regulation in *Escherichia coli*. *Mol Microbiol* **6:** 1913–1923.

Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. (2000) NPS@: network protein sequence analysis. *Trends Biochem Sci* **25:** 147–150.

Conrad, R. (1996) Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiol Rev* **60:** 609–640.

Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16:** 10881–10890.

DiSpirito, A.A., Taaffe, L.R., Lipscomb, J.D., and Hooper, A.B. (1985) A 'blue' copper oxidase from *Nitrosomonas europaea*. *Biochim Biophys Acta* **827:** 320–326.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77:** 7347–7351.

Dodd, I.B., and Egan, J.B. (1990) Improved detection of helix–turn–helix DNA-binding motifs in protein sequences. *Nucleic Acids Res* **18:** 5019–5026.

Gerhus, E., Steinrucke, P., and Ludwig, B. (1990) *Paracoccus denitrificans* cytochrome c_1 gene replacement mutants. *J Bacteriol* **172:** 2392–2400.

Hommes, N.G., Sayavedra-Soto, L.A., and Arp, D.J. (1996) Mutagenesis of hydroxylamine oxidoreductase in

Nitrosomonas europaea by transformation and recombination. *J Bacteriol* **178:** 3710–3714.

Hooper, A.B. (1968) A nitrite-reducing enzyme from *Nitrosomonas europaea*. Preliminary characterization with hydroxylamine as electron donor. *Biochim Biophys Acta* **162:** 49–65.

Hyman, M.R., and Arp, D.J. (1992) $^{14}C_2H_2$- and $^{14}CO_2$-labeling studies of the *de novo* synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. *J Biol Chem* **267:** 1534–1545.

Hynes, R.K., and Knowles, G. (1984) Production of nitrous oxide by *Nitrosomonas europaea*: effects of acetylene, pH, and oxygen. *Can J Mircrobiol* **30:** 1397–1404.

Jain, R., and Shapleigh, J.P. (2001) Characterization of *nirV* and a gene encoding a novel pseudoazurin in *Rhodobacter sphaeroides* 2.4.3. *Microbiology* **147:** 2505–2515.

Jiang, Q.Q., and Bakken, L.R. (1999) Nitrous oxide production and methane oxidation by different ammonia-oxidizing bacteria. *Appl Environ Microbiol* **65:** 2679–2684.

Keon, R.G., Fu, R., and Voordouw, G. (1997) Deletion of two downstream genes alters expression of the *hmc* operon of *Desulfovibrio vulgaris subsp. vulgaris Hildenborough*. *Arch Microbiol* **167:** 376–383.

Kester, R.A., de Boer, W., and Laanbroek, H.J. (1997) Production of NO and N_2O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration. *Appl Environ Microbiol* **63:** 3872–3877.

Kwiatkowski, A.V., and Shapleigh, J.P. (1996) Requirement of nitric oxide for induction of genes whose products are involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. *J Biol Chem* **271:** 24382–24388.

Madigan, M.T., Martinko, J.M., and Parker, J. (2000) *Brock Biology of Microorganisms.* Upper Saddle River, NJ: Prentice Hall.

Maeda, S., Okamura, M., Kobayashi, M., and Omata, T. (1998) Nitrite-specific active transport system of the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **180:** 6761–6763.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, D.J., and Nicholas, D.J. (1985) Characterization of a soluble cytochrome oxidase/nitrite reductase from *Nitrosomonas europaea*. *J Gen Microbiol* **131:** 2851–2854.

Nicholas, D.J., and Nason, A. (1957) Determination of nitrate and nitrite. *Methods Enzymol* **3:** 981–984.

Poth, M., and Focht, D.D. (1985) ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. *Appl Environ Microbiol* **49:** 1134–1141.

Remde, A., and Conrad, R. (1990) Production of nitric oxide in *Nitrosomonas europaea* by reduction of nitrite. *Arch Microbiol* **154:** 187–191.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Saunders, N.F., Houben, E.N., Koefoed, S., de Weert, S., Reijnders, W.N., Westerhoff, H.V., *et al.* (1999) Transcription regulation of the *nir* gene cluster encoding nitrite reductase of *Paracoccus denitrificans* involves NNR and NirI, a novel type of membrane protein. *Mol Microbiol* **34:** 24–36.

Simon, R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1:** 37–45.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtenberg, B.J.J. (1987) Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9:** 27–39.

van Spanning, R.J., De Boer, A.P., Slotboom, D.J., Reijnders, W.N., and Stouthamer, A.H. (1995) Isolation and characterization of a novel insertion sequence element, IS1248, in *Paracoccus denitrificans*. *Plasmid* **34:** 11–21.

Stein, L.Y., and Arp, D.J. (1998) Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. *Appl Environ Microbiol* **64:** 4098–4102.

Stewart, V., and Parales, J., Jr (1988) Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. *J Bacteriol* **170:** 1589–1597.

Stewart, V., Chen, L.L., and Wu, H.C. (2003) Response to culture aeration mediated by the nitrate and nitrite sensor NarQ of *Escherichia coli* K-12. *Mol Microbiol* **50:** 1391–1399.

Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., *et al.* (2003) The COG database: an updated version includes eukaryotes. *BMC Bioinform* **4:** 41.

Todd, J.D., Wexler, M., Sawers, G., Yeoman, K.H., Poole, P.S., and Johnston, A.W. (2002) RirA, an iron-responsive regulator in the symbiotic bacterium *Rhizobium leguminosarum*. *Microbiology* **148:** 4059–4071.

Whittaker, M., Bergmann, D., Arciero, D., and Hooper, A.B. (2000) Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium *Nitrosomonas europaea*. *Biochim Biophys Acta* **1459:** 346–355.

Winogradsky, S. (1892) Contributions a la morphologie des organisms de la nitrification. *Arch Sci Biol (St. Petersb.)* **1:** 88–137.

Wood, N.J., Alizadeh, T., Bennett, S., Pearce, J., Ferguson, S.J., Richardson, D.J., and Moir, J.W. (2001) Maximal expression of membrane-bound nitrate reductase in *Paracoccus* is induced by nitrate via a third FNR-like regulator named NarR. *J Bacteriol* **183:** 3606–3613.

Wood, N.J., Alizadeh, T., Richardson, D.J., Ferguson, S.J., and Moir, J.W. (2002) Two domains of a dual-function NarK protein are required for nitrate uptake, the first step of denitrification in *Paracoccus pantotrophus*. *Mol Microbiol* **44:** 157–170.

Wu, Q., and Stewart, V. (1998) NasFED proteins mediate assimilatory nitrate and nitrite transport in *Klebsiella oxytoca* (*pneumoniae*) M5al. *J Bacteriol* **180:** 1311–1322.

Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61:** 533–616.